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A STUDY OF THE NUTRITIONAL VALUE OF CEREAL GRAIN PROTEINS

by

William Lobay

Department of Animal Science

UNIVERSITY OF ALBERTA

April 1947

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The undersigned hereby certify that they have read and recommend to the Committee on Graduate Studies for acceptance a thesis on "A Study of the Nutritional Value of Cereal Grain Proteins" submitted by William Tobay, B.Sc., in partial fulfillment of the requirements for the degree of Master of Science.

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I. Feeding Experiments with Grains of Different Protein Content.

II. Amino Acid Assays on Grains.

William Lobay

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A THESIS

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Thesis
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ACKNOWLEDGMENTS

I am particularly indebted to Dr. L. W. McElroy, Associate Professor of Animal Husbandry, who initiated and supervised the study herein reported, and who through helpful criticisms and suggestions rendered much valuable and appreciated assistance in carrying out this research, and in the preparation of this manuscript. Thanks and appreciation are also extended to Professor J. P. Sackville, Head of the Department of Animal Science, for placing the facilities of his department at my disposal, and to Dr. G. Hunter, Head of the Department of Biochemistry, for providing the test animals and the space for conducting feeding trials. The very valuable advice received from Mr. D. R. Clandinin, Assistant Professor of Poultry, regarding the conduction of microbiological assays, is gratefully acknowledged.

This investigation was made possible through financial assistance from the Committee on Agricultural Research Grants, Project 4.

TABLE OF CONTENTS

	Page
INTRODUCTION	1
PART I - FEEDING EXPERIMENTS WITH GRAINS OF DIFFERENT PROTEIN CONTENT	2
Review of Literature	2
Experiments at the University of Alberta	8
Experiment 1	8
Experiment 2	17
Experiment 3	21
Experiment 4	27
Summary	32
PART II - AMINO ACID ASSAYS ON GRAINS	34
Review of Literature	35
Assay methods	35
Hydrolysis methods	37
Experimental	38
Assay materials	38
Organism and Inoculum	38
Basal Medium	39
Preparation of Samples for Assay	44
Assay Procedure	45
Preparation of Standards	46
Validity of Procedure	47

TABLE OF CONTENTS (continued)

	Page
Validity of Procedure	47
Hydrolysis Agents	47
Duration of Autoclaving in 2 N HCl	49
Losses of Amino Acids in Humin	50
Salt Inhibitory Effects	51
Reproducibility	52
Recovery Experiments	53
Results and Discussion	55
Summary	64
Literature Cited	67

A STUDY OF THE NUTRITIONAL VALUE OF CEREAL GRAIN PROTEINS.

INTRODUCTION

The importance of knowledge regarding the nutritional adequacy of cereal grain proteins is apparent from the fact that the cereal grains constitute a major source of dietary protein for both humans and domestic animals. In any attempt to appraise the nutritional value of a feed with respect to protein, both the quantity and quality of the protein in that food must be considered.

Ample analytical work has been done to show that marked differences may exist in the protein content of any given variety of cereal grain, depending upon the conditions of the soil and climate under which the crop is grown. As an extreme example, barley grown in the gray soil belt north and west of Edmonton may have a crude protein as low as 7 to 8% as compared to 15 to 16% for the same variety grown in the south-east section of the province. Such wide variations in total protein suggest the possibility of significant differences, from the point of view of protein nutrition, between grains grown in different areas, but no adequate data are available from either feeding trials or analytical studies of the amino acid composition of these grains to permit accurate assessment of their relative feeding value.

Experiments undertaken to provide data which may be useful in appraising the nutritional value of grain proteins are discussed in this thesis. In Part I feeding experiments conducted with grains of different protein content are considered, while microbiological amino acid assays on grains are described in Part II.

PART I - FEEDING EXPERIMENTS WITH GRAINS OF DIFFERENT PROTEIN CONTENT

REVIEW OF LITERATURE

One of the earliest works on this continent relative to the nutritive merits of cereal grain proteins was that of Osborne and Mendel in 1912(1). Using rye, barley and wheat proteins as the only source of protein, they showed the inability of the rat to grow on an otherwise adequate diet. In their classical experiment (2) these workers demonstrated that the proteins gliadin and zein are nutritionally inadequate for the rat but may be rendered satisfactory by the addition of missing amino acids. On an otherwise adequate diet, but containing zein as the only source of protein, rats failed to grow and lost weight. The addition of lysine and tryptophane to the diet resulted in resumption of growth.

Experiments of this nature stimulated immediate interest. In 1914 Hart and McCollum (3) demonstrated growth responses significantly below normal when wheat, fortified with vitamins and minerals, was fed to swine and rats but that normal growth was obtained when this ration was supplemented with casein. In 1917 McCollum, Simmonds and Pitz (4) concluded from experiments involving a small number of rats that the oat kernel contains proteins of poorer quality than those furnished by maize or wheat. Hogan (5) in the same year concluded that the proteins of corn are deficient in tryptophane, and when this amino acid is supplied, a lysine deficiency is manifested. These and other experiments established quite early the fact that cereal grains are lacking in certain amino acids which cannot be synthesized by the animal body but must be supplied preformed.

There are very few references in the literature in regard to feeding experiments with grains of different protein content. In two out of three trials, Wright (7) of South Dakota found that light barley of high protein content gave smaller daily gains in pigs than did heavy barley of low protein content. By contrast, in experiments conducted at the University of Alberta in 1945 (8), mixtures made up

of oats and barley containing 12.5 - 16.5% protein gave more rapid growth in hogs than did mixtures made up of oats and barley containing approximately 9% protein. Similar results were obtained in a second experiment completed in 1946 (9).

One of the first experiments involving supplementation of whole grains with amino acids was that of Mitchell and Smuts reported in 1932 (10). The addition of pure lysine to wheat, corn or oats in an amount furnishing 0.25% concentration in the ration resulted in large increases in the growth promoting value of these grains for the rats. In 1940 Kik (11), in a series of paired feeding experiments with rats, added cystine, methionine, lysine and tryptophane singly to whole and polished rice. Cystine, methionine and lysine were effective supplements but tryptophane failed to produce any improvement in growth.

In 1940 Marias and Smuts (12) reported that supplementation of corn with either lysine or tryptophane alone failed to improve the utilization of nitrogen by the rat, whereas marked improvement was obtained when these two amino acids were added in combination. In the same paper evidence is presented to show that the proteins of white corn are significantly better than those of the yellow variety. Csonka and Jones (13) have shown that the cystine, tryptophane and tyrosine content of the protein of soybeans varies in different varieties.

The possibility of supplementing cereal grains with

lysine on a commercial basis has been suggested by Crampton (14). Feeding experiments with pigs following his proposed plan in which the grain ration would be fortified with lysine in order to provide a balanced amino acid pattern, have not been published.

In order to estimate the quantity of pure amino acids which it would be necessary to add to a grain ration to meet amino acid requirements, the amino acid composition of the grains and the minimum amino acid requirements of the animal must be known. The best data available in the literature (15 - 20) regarding the amino acid composition of grain proteins are summarized in Table I. The amino acid composition of casein, a protein of high biological value, is included for comparative purposes.

Table I

Approximate Percentage of Amino Acids in Casein and Some Plant Proteins

Amino Acid	Casein[15]	Corn[15]	Wheat[15]	Oats[17]	Barley[20]	Rye[16]
		% of total protein				
Arginine	4.1	4.0	3.0	6.0	4.8	4.3
Histidine	2.5	2.4	1-2	2.0	2.1	1.72
Lysine	6.9	2.5	2.7	3.3	1.8	4.2
Tyrosine	6.4	6.1	3.8	4.5		
Trypto-phane	1.5	0.6	1.0	1.3		1.31
Phenyl-alanine	5.2	4-5	5.7	6.9		5.6
Cystine	0.4	1.1	1.3	1.8		
Methionine	3.5	4.0	3.0	2.3		1.26
Threonine	3.9	3.6	3.3	3.5		3.0
Leucine	12.0	22.0	5.8[17]	8.0		6.2
Isoleucine	5.0	3-4	4	5.3		4.0
Valine	7.0	5.0	3-4	6.5		5.0
Glycine	0.5					

The only species for which the amino acid requirements have been studied in any detail are the human (15, 21), the chick (22, 23) and the rat (24, 25). Amino acid requirements of the rat, as summarized by Rose (24) are listed in Table II.

Table II

Essential Amino Acids and the Minimum Requirements Necessary to Support Normal Growth in Rats when the Nonessentials are Included in the Diet

Amino Acid	Percent of Total Food Consumed
1. Lysine	1.0
2. Leucine	0.9
3. Valine	0.7
4. Phenylalanine	0.7
5. Methionine	0.6
6. Threonine	0.6
7. Isoleucine	0.5
8. Histidine	0.4
9. Tryptophane	0.2
10. Arginine	0.2

Calculations from the data given in Tables I and II show that the cereal grains would supply about one-third of the lysine and from 50 to 75 per cent of the methionine, tryptophane, histidine and threonine required by the rat for normal growth. Leucine, valine and phenylalanine would be furnished in marginal amounts, while isoleucine and arginine would be present in excess of the amount required.

EXPERIMENTS AT THE UNIVERSITY OF ALBERTA

The experiments reported in this section were carried on at the University of Alberta in 1945 and 1946. They were designed to study:

(1) the comparative growth response of rats fed grains of markedly different protein content.

(2) the effect of adding pure amino acids to a basal ration of low protein barley.

The Comparative Growth Response of Rats fed Grains of Markedly Different Protein Content.

It has been mentioned that there are significant differences in the protein content of the same grains grown on different types of soil and under different climatic conditions. Experiment I was initiated as a pilot experiment to test the validity of the assumption that the same grains of different protein content have different nutritive value for the rat.

EXPERIMENT I

Objects of the Experiment:

(1) To study the comparative nutritional value for rats of high and low protein wheat, barley and oats, with grain as the only source of protein.

(2) To study the effect of feeding a mixture of oats and barley as compared to feeding these grains singly.

(3) To compare the growth response of rats fed low protein barley supplemented with casein with that of rats fed grain as the only source of protein.

Grains Used

The grains used, together with their protein content and the soil zone in which they were grown, are shown in Table III.

Table III

Grains Used in Experiment I

Grain	Protein %	Soil Zone
Low protein oats	8.2	Gray Transitional
High protein oats	15.9	Brown
Low protein barley	9.4	Gray Transitional
High protein barley	16.2	Brown
Low protein wheat	8.9	Gray
High protein wheat	17.3	Brown

Experimental Animals

Weanling white rats from the University of Alberta, Department of Biochemistry rat colony were used as test animals. Fifty-four white rats were divided into nine groups of six rats each, and were allotted as uniformly as possible with respect to breeding, age, sex and weight.

Test groups

The test group numbers and the rations fed were designated as follows:

Group No.	Ration
1.	Low protein oats.
2.	High protein oats.
3.	Low protein barley.
4.	High protein barley.
5.	Low protein wheat.
6.	High protein wheat
7.	Low protein oats and barley, equal parts.
8.	High protein oats and barley, equal parts.
9.	Low protein barley + 8% vitamin-free casein.

Rations

With the exception of the ration for group 9 the only source of dietary protein was grain. All rations were supplemented with fat, minerals and vitamins in amounts estimated to be adequate to support normal growth in rats, so that any difference between groups in growth response could be attributed primarily to differences in the amount, or the amount and quality, of protein in the grain used in the ration.

Details of the ration are shown below:

(a) Basal ration

Basal mixture for groups 1 to 8.

Grain*	930 grams
Crisco	30 grams
Salt mixture**	40 grams
Total	1000 grams

Basal mixture for group 9.

Grain*	850 grams
Vitamin-free casein	80 grams
Crisco	30 grams
Salt mixture**	40 grams
Total	1000 grams

---

* All grains were ground in a Wiley mill to pass through a 1 mm. screen mesh.

** McCollum's salt mixture (26):

Calcium lactate ($Ca\left[CH_3.CH(OH).COO\right]_2.5H_2O$)	352 grams
Calcium phosphate ($Ca(H_2PO_4).H_2O$)	146 grams
Potassium acid phosphate (KH_2PO_4)	258 grams
Sodium acid phosphate ($NaH_2HPO_4.H_2O$)	94 grams
Sodium chloride ($NaCl$)	47 grams
Magnesium sulphate ($MgSO_4.7H_2O$)	147 grams
Iron citrate	32 grams
Total	1076 grams

(b) Vitamin Supplements

The following vitamin supplements were added to each kilogram of basal mixture:

Fish oil (3000A - 400D)	3 grams
Thiamine	10 milligrams
Riboflavin	20 "
Pyridoxine	10 "
Pantothenic acid	20 "
Nicotinic acid	10 "
Choline chloride	1 gram
Biotin	10 micrograms

In addition each rat was dosed orally once a week with 2 milligrams of alpha tocopherol in peanut oil and 2 drops of fish oil.

Housing, Feeding and Weighing

All rats were housed in individual wire screen cages. Food and water were supplied to each rat ad libitum, daily. All the animals were weighed once a week.

Results and Discussion of Experiment I

The results of this trial are summarized in Figure 1 and Table IV.

EXPERIMENT 1

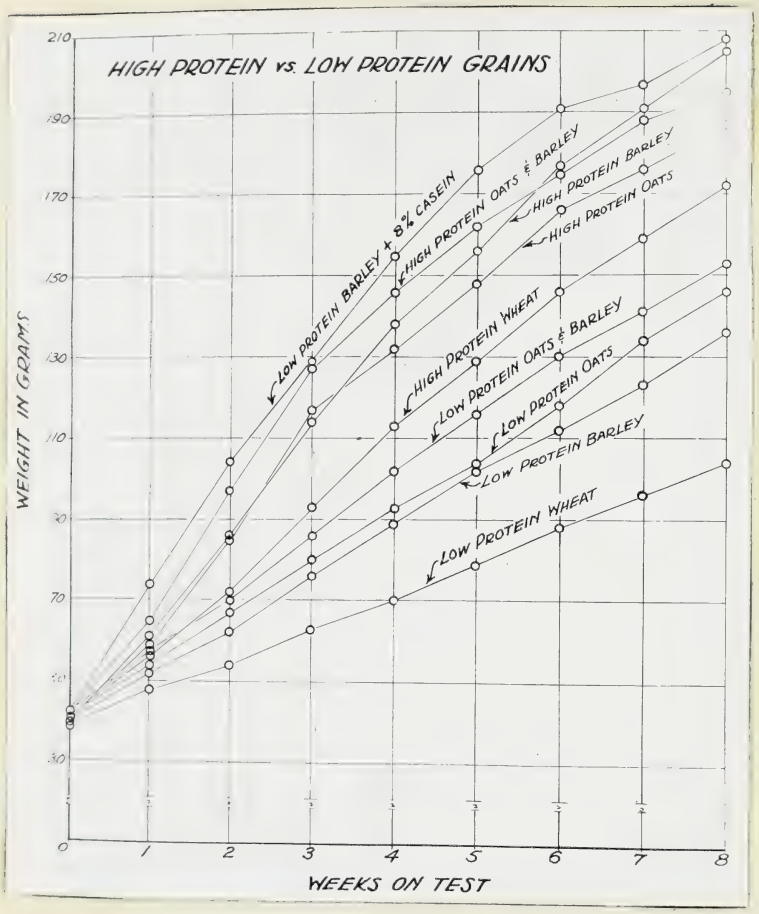

Fig. 1. Growth curves of weanling rats fed grains of markedly different protein content.

Table IV

Growth Response of Rats Fed Grains of Different Protein Content. (Trial: 56 days)

Group No.	Av. Initial Wt. (Grams)	Av. Final Wt. (Grams)	Av. Total Gain per Rat (Grams)	Av. Daily Gain (Grams)
1 L.P.O.	41	146	105	1.87
2 H.P.O.	40	187	147	2.62
3 L.P.B.	40	136	96	1.71
4 H.P.B.	40	205	165	2.95
5 L.P.W.	39	104	65	1.16
6 H.P.W.	41	172	131	2.34
7 L.P.O.+ B.	42	153	111	1.98
8 H.P.O.+ B.	42	195	153	2.73
9 L.P.B. + 8% casein	42	208	166	2.96

It is evident that in every case the rate of gain was markedly in favor of high protein grains. In addition the rats in the groups fed high protein grain were more active and sleek in appearance (see plate 1, page 65) than the rats in the groups fed low protein grain. This indicates the lower nutritional value of the low protein grains.

Rats fed barley containing 16.2% protein grew at practically the same rate as those fed barley containing 9.4% protein but supplemented with 8% vitamin-free casein. Since casein is a protein of high biological value, this suggests that the combination of amino acids in the high protein barley

1	[illegible]	[illegible]	[illegible]	[illegible]
2	[illegible]	[illegible]	[illegible]	[illegible]
3	[illegible]	[illegible]	[illegible]	[illegible]
4	[illegible]	[illegible]	[illegible]	[illegible]
5	[illegible]	79	149	69
6	[illegible]	81	179	161
7	[illegible]	[illegible]	[illegible]	[illegible]
8	[illegible]	82	185	119
9	[illegible]	82	205	119

was such as to make it a reasonably high quality protein for rats.

The results indicate that the proteins of oats and barley do not supplement each other to any marked degree. This was anticipated and is in agreement with available reports that cereal proteins tend to be deficient in the same essential amino acids (17, 15).

It is of interest to note that up to the eighth week on test, growth on the wheat rations was much poorer than on oat or barley rations of comparable protein content.

If the high protein barley ration fed to group 2 is assigned a nutritional value of 100, the grains fed to the remaining groups could be evaluated as shown below on the basis of the comparative rates of gain when they were fed in this trial:

Low protein barley + 8% casein	100.3
High protein barley	100
High protein oats and barley, equal parts	93
High protein oats	89
High protein wheat	79
Low protein oats and barley, equal parts	67
Low protein oats	63
Low protein barley	58
Low protein wheat	39

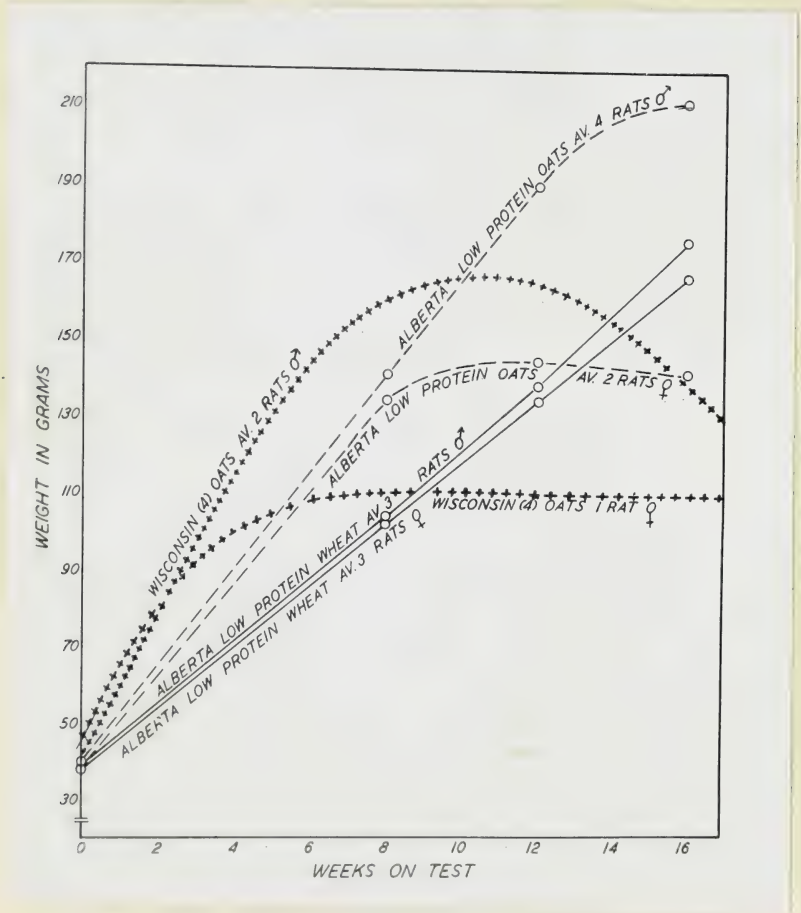

Fig. 2. Growth of rats on oat rations - Wisconsin results, 1917, vs. Alberta results, 1946.

With regard to the inferior growth response of rats fed wheat it is of interest to recall that investigators (4) at the University of Wisconsin have concluded that the protein of the oat kernel is inferior to that of wheat. This conclusion was based on the experiments in which it was found that, although rats fed an oat ration supplemented with minerals and vitamins made reasonably good gains in weight for the first two months on test, growth ceased, and in some cases the rats lost weight, during the third and fourth months.

A number of the rats from Alberta Experiment I were kept on test for 16 weeks. Growth curves for the rats fed low protein oats and those fed low protein wheat are shown in Figure 2. Corresponding growth curves for rats fed an oat ration in the Wisconsin experiments (4) are included for comparison. It will be noted that in the Alberta experiment the average growth curve for the two female rats fed oats levelled off after the tenth week at approximately 145 grams. In the Wisconsin experiment the single female failed to grow after attaining a weight of 110 grams at the end of the seventh week.

The growth curves for male rats fed oats are quite different in the Alberta and Wisconsin trials. In the Wisconsin trial male rats lost weight after the eleventh week, while in the Alberta experiment the growth curve for male rats began to plateau at approximately 210 grams after sixteen weeks on test.

Growth curves for rats fed wheat in the Wisconsin experiments (27) are not shown in Figure 2, but they do not

differ materially from those shown for the Alberta experiment.

The number of test animals used in these experiments was small and the significance of the Wisconsin results may be further questioned in view of the fact that the vitamin supplements used in 1917 may not have been adequate. Nevertheless the question of the relative adequacy of wheat and oat proteins for the growing rat seems worthy of further study, particularly in view of the fact that both experiments suggest the possibility of a sex difference.

The Effect of Adding Pure Amino Acids to a Basal Ration of Low Protein Barley.

The previous experiment showed that low protein barley was much less effective than high protein barley in promoting growth in rats. It has also been found from the results of a trial with pigs (8) that feeding a mixture of low protein (±9%) oats and barley resulted in significantly lower rates of gain than were obtained with mixtures of medium (± 12%) or high (± 16%) protein oats and barley. Much of the low protein barley from the gray-wooded soils is normally marketed through hogs, and in the light of these results it would appear that the question of satisfactory protein supplements for swine in gray-wooded soil areas may be one that deserves special study. With this thought in mind low protein barley was selected as the test

grain for rat feeding experiments involving the supplementation of grain with pure amino acids.

EXPERIMENT 2

Object of the Experiment

(1) To compare the growth response of rats fed a basal ration of low protein barley with that of rats fed this basal ration supplemented with pure amino acids or with casein.

Experimental Material

The low protein (9.4%) barley used was from the same batch as that used in Experiment 1. The rats were from the same source as those in the first trial and the same experimental procedure was followed except that daily food consumption records were kept and the animals were weighed every second day. The rats were started on test at 21 days of age. Four groups of three rats were made up by assigning one litter mate from each of three litters to a separate group.

Test Groups

Group No.	Ration
1.	Low protein barley.
2.	Low protein barley + 8% vitamin-free casein.
3.	Low protein barley plus an amount of the amino acid lysine calculated to be equivalent to that supplied by 8% casein.

4. Low protein barley + 10 essential amino acids in an amount calculated to be equivalent to that supplied by 8% casein.

Rations

The rations were compounded from grain, Crisco, salt mixture and vitamin supplement as described in Experiment 1. The values reported by Block and Bolling (17) were used to estimate the amounts of each essential amino acid supplied by 8% casein.

Rose (25) has shown that only the natural forms of valine, leucine, isoleucine, lysine and threonine are utilized by the rat. The natural forms of tryptophane, histidine, phenylalanine and methionine can be replaced by their antipodes for growth purposes although d-histidine appears to be somewhat less efficient than l-histidine. The position of arginine is not definitely known.

The amino acid isomers used and the quantities added to the basal ration to make 250 grams are shown in Table V.

Table V

Isomeric Forms and Amounts of Amino Acids Added to the Basal Ration.

Amino Acid	Natural Isomer	Amt. Calculated equiv. 8% casein (grams)	Isomer Used	Amount Added (grams)
Lysine	l(+)	1.38	l(+)***	1.72
Leucine	l(-)	2.40	l(-)	2.40
Phenylalanine*	l(-)	1.04	dl	1.04
Valine	l(+)	1.40	dl**	2.80
Threonine	d(-)	0.78	dl**	1.56
Methionine*	l(-)	0.70	dl	0.70
Isoleucine	l(+)	1.00	dl**	2.00
Histidine*	l(-)	0.50	l(+)***	0.58
Tryptophane*	l(-)	0.30	l(-)	0.30
Arginine	l(+)	0.82	l(+)***	0.99

The amino acids were finely ground in a mortar and mixed thoroughly with the grain. Appropriate amounts of other ingredients were then added and the whole ration was put through a wire sieve several times.

Results and Discussion of Experiment 2

The results of this trial are summarized in Figure 3 and Table VI.

* Unnatural isomer can replace the natural form.

** Unnatural isomer cannot replace the natural form.

*** Monohydrochloride.

EXPERIMENT 2

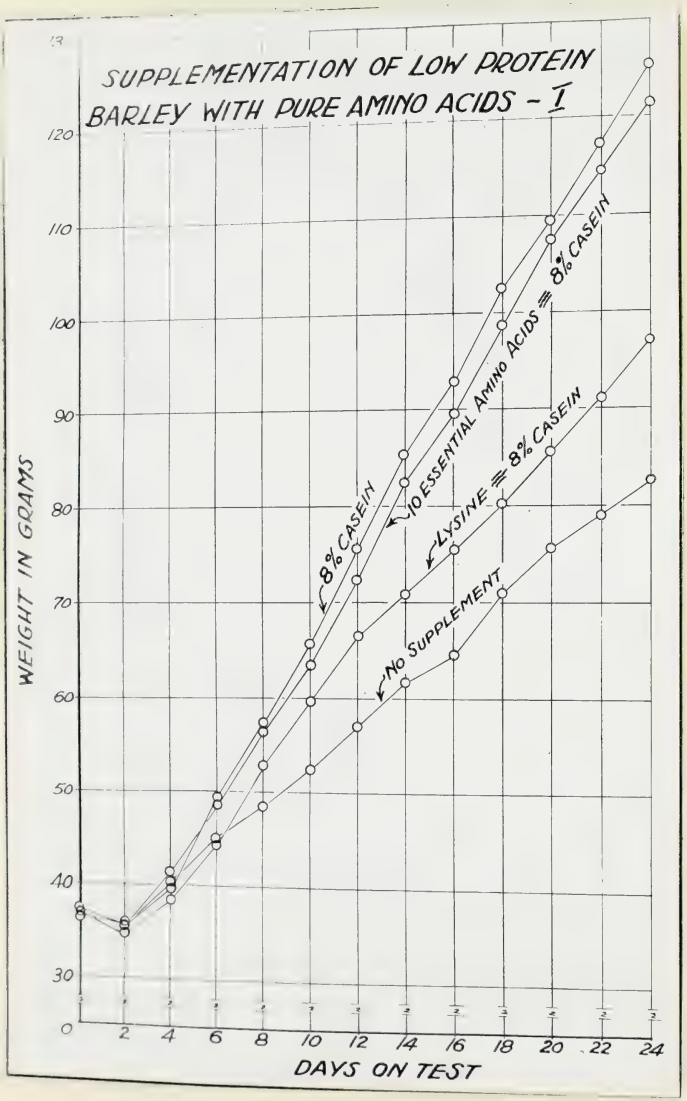

Fig. 3. Growth curves of weanling rats.

Table VI

The Effect of Adding Pure Amino Acids to a Basal Ration of Low Protein Barley

	Group Number			
	1.	2.	3.	4.
	Basal	8% casein	Lysine	10 E.A.A.
No. of rats in group	3	3	3	3
No. days in period	24	24	24	24
Av. initial wt. in grams	36.7	36.7	37.0	37.0
Av. final wt. " "	82.8	125.3	97.0	121.3
Total gain " "	46.1	88.6	60.0	84.3
Av. daily gain " "	1.92	3.69	2.50	3.51
Av. daily feed " "	8.3	9.4	9.1	9.6
Feed consumed per gram gain (grams)	4.3	2.5	3.6	2.7

A supplement made up of the ten essential amino acids was almost as effective as a supplement of 8% vitamin-free casein as measured by the rate of growth and economy of utilization of feed by the rats in groups 2 and 4.

If the ration of low protein barley supplemented with 8% casein is assigned a nutritional value of 100, the remaining rations could be evaluated as shown below:

Low protein barley + 8% casein	100
" " " + 10 E.A.A.	95
" " " + lysine	77
Low protein barley	60

It is of interest to note that while the addition of ten essential amino acids gave a 35 point increase over the basal diet, lysine alone contributed 17 points to this increase. This is in agreement with evidence in the literature that lysine is the chief limiting amino acid in the cereal grains (15, 17).

The rats fed rations supplemented with lysine or the ten essential amino acids were more active, more firmly fleshed and better in general appearance (see Plates 2 and 3, page 66) than those fed the basal ration.

There was a temporary inhibition in growth in all groups during the first two days of the experiment. Gunther and Rose (28) have observed a similar inhibition when the animals were transferred from the stock ration to one containing mixtures of amino acids, a method employed in this experiment.

The animals fed the basal ration consumed approximately 1 gram less of their diet daily than those in other groups. Windus, Catherwood and Rose (29) state that, "in general the better the nutritive quality of the food, as indicated by the growth rate, the greater is the food consumption. Unquestionably, rats will consume adequate amounts of diets .....when all necessary components are present in the food."

EXPERIMENT 3

In Experiment 2 a supplement of the ten essential amino acids gave an 18 point increase in growth over that obtained

with a supplement of lysine alone. Which of the nine remaining essential amino acids contributed most to this increase was not known. Efforts to calculate the degree of deficiency which might exist in barley with respect to each of these amino acids were hindered by the fact that barley assay values were available for only arginine, histidine and lysine. For the remaining seven amino acids it was therefore necessary to apply wheat and oat assay results to barley. On the basis of an estimate of the amino acid composition of barley obtained in this way and using the data of Rose (24) for the amino acid requirements of the rat, the figures shown in Table VII were obtained.

Table VII

Calculated Daily Amino Acid Requirement as Compared to Calculated Daily Intake of Rats Consuming 10 Grams of Barley per Day

Amino Acid	Requirements per day (mg.)	Amount in barley (mg.)	% Requirement Supplied by barley
Lysine	100	14	14
Leucine	90	116	129
Phenylalanine	70	51	73
Valine	70	30	43
Threonine	60	30	50
Methionine	60	30	50
Isoleucine	50	36	72
Histidine	40	23	58
Tryptophane	20	9	45
Arginine	20	38	190

The approximations shown in Table VII suggest that after lysine the amino acids valine, tryptophane, methionine and threonine may be the chief limiting amino acids in barley protein. Experiment 3 was therefore designed with the following objectives in mind:

(1) To study the effectiveness of a supplement of lysine and tryptophane when added to a basal ration of low protein barley.

(2) To study the effectiveness of a supplement made up of the five essential amino acids, lysine, tryptophane, methionine, threonine and valine when added to a basal ration of low protein barley.

Experimental Material

The grain used and the source, allotment, housing, feeding and weighing of rats were the same as in Experiment 2. The method for calculations of quantities of the amino acid supplement to be used and the preparation of the rations was the same as in Experiment 2.

Test Groups

Group No.	Ration
1.	Low protein barley.
2.	Low protein barley + 8% casein.
3.	Low protein barley + lysine calculated to be equivalent to that supplied by 8% casein.
4.	Low protein barley + lysine and tryptophane equivalent to that supplied by 8% casein.

The abbreviations shown in Table V are explained in the text.

and thereafter may be the chief limiting factor in barley production. Experiment 2 was therefore designed with the following objectives in mind:

(1) To study the effectiveness of a supplement of lysine and tryptophane when added to a basal ration of low protein barley.

(2) To study the effectiveness of a supplement containing one of the five essential amino acids, lysine, tryptophane, methionine, threonine and valine when added to a basal ration of low protein barley.

Experimental Material

The grain used was the variety, Albatross, locally grown. Feeding and weighing of rats were the same as in Experiment 1. The method for calculations of quantities of the amino acid supplement to be used and the preparation of the ration was the same as in Experiment 2.

Table I.

1.	low protein barley.
2.	low protein barley + DL methionine.
3.	low protein barley + lysine sulphate.
4.	low protein barley + lysine and tryptophane in amounts equivalent to those supplied by 5% casein.
5.	low protein barley + lysine and tryptophane in amounts equivalent to those supplied by 10% casein.

5. Low protein barley + lysine, tryptophane, methionine, threonine and valine equivalent to that supplied by 8% casein.

Results

The average growth response of the rats in the different groups during the 22-day period of the trial is shown in Figure 4 and Table VIII.

Table VIII

Growth Response of Rats Fed the Basal Ration Supplemented with Amino Acids

	Group Number				
	1.	2.	3.	4.	5.
	Basal	8% Casein	Lysine	Lysine + trypto-phane	5 E.A.A.
No. of rats in group	3	3	3	3	3
No. days in period	22	22	22	22	22
Av. Initial wt.*(grams)	40.2	40.5	40.0	39.2	38.5
Av. final wt. "	80.7	127.0	101.3	90.7	90.0
Total gain "	40.5	86.5	61.3	51.5	51.5
Av. daily gain "	1.84	3.93	2.79	2.34	2.34
Av. daily feed "	9.5	11.2	10.2	9.5	8.4
Feed consumed per gram gain (grams)	5.2	2.8	3.7	4.0	3.6

* All rats kept on basal ration for two days prior to experiment.

7. [illegible] new [illegible] Double + Lysine, [illegible]
[illegible], Threonine and [illegible]
to that supplied by [illegible].

[illegible]

The average growth response of the pigs in [illegible]
groups during the 12-day period of the trial is shown in
Figure 4 and Table VIII.

[illegible]

Growth Response of Pigs fed the Basal Ration
[illegible] Supplemented with Amino Acids

[illegible]	1.	2.	3.	4.	5.
No. [illegible]					
No. days in period	12	12	12	12	12
Av. [illegible] wt.					
[illegible]					
[illegible]					
[illegible]					
Feed consumed per [illegible]					

* All pigs [illegible]

[illegible]

EXPERIMENT 3

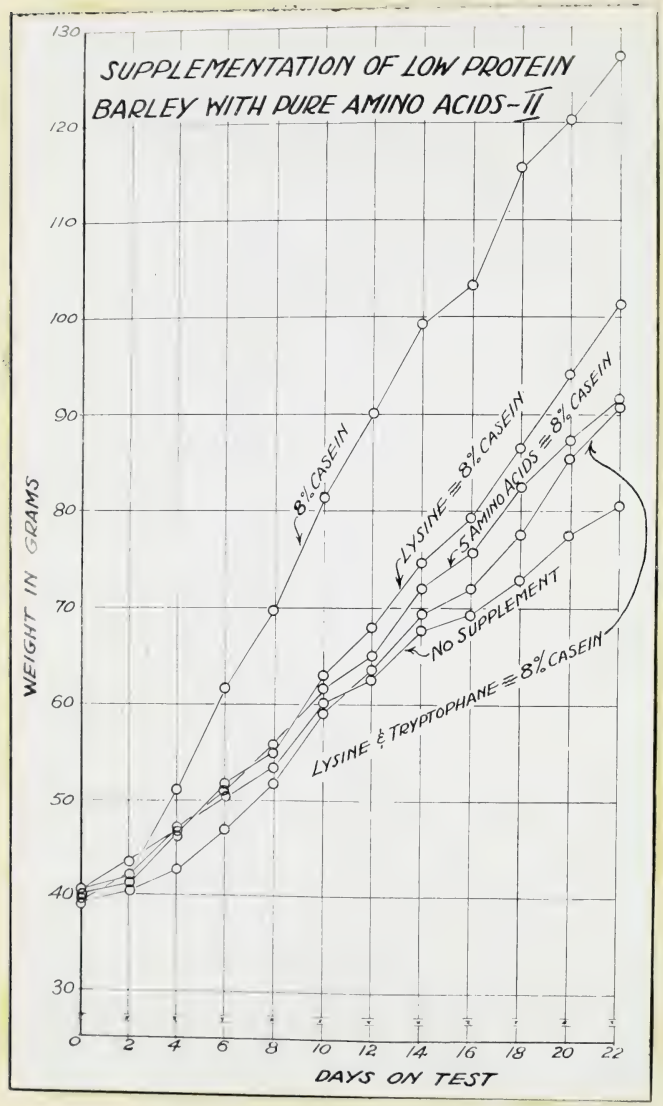

Fig. 4. Growth curves of weanling rats.

The growth responses of rats on the basal diet and the basal supplemented with 8% casein were similar to those of Experiment 2, while the basal ration supplemented with lysine gave a slightly better response than in the previous trial.

As compared to lysine alone supplemental tryptophane, methionine, threonine and valine along with lysine appeared to be detrimental. Similarly a combination of lysine and tryptophane was not as effective in promoting growth as was lysine alone.

If the ration of low protein barley plus 8% casein is assigned a nutritional value of 100, the remaining rations may be ranked as shown below:

Low protein barley + 8% casein	100
Low protein barley + lysine	80
Low protein barley + 5 essential amino acids	70
Low protein barley + lysine and tryptophane	70
Low protein barley	53

Discussion

Since the addition of other amino acids with lysine incorporated in the diet did not show the stimulatory effect anticipated, the experiment was repeated. The results are shown in Table IX. Due to the lack of sufficient lysine, the lysine plus tryptophane group was omitted.

Table IX

Growth Response of Rats Fed the Basal Ration Supplemented with Amino Acids

	Group Number			
	1.	2.	3.	4.
	Basal	8% Casein	Lysine	5 E.A.A.
No. of rats in group	3	3	3	3
No. days in period	14	14	14	14
Av. initial wt. (grams)*	36.3	35.7	37.0	35.3
Av. final wt. "	52.8	89.2	61.3	45.2
Total gain "	16.5	53.5	24.3	9.9
Av. daily gain "	1.18	3.82	1.73	0.45
Av. daily feed "	5.57	7.24	6.50	3.40

Since the available supply of amino acids was limited this trial was discontinued at 14 days when it was evident that the growth response from the five amino acids was even less than that obtained in the preceding trial (Experiment 3). The comparable figures for average daily gain up to the fourteenth day in Experiment 3 were: basal, 1.84 grams; 8% casein, 3.93 grams; lysine, 2.34 grams, and five amino acids, 2.34 grams. It is not clear why a supplement of lysine alone should give better growth than a supplement of lysine, tryptophane, methionine, threonine and valine. This question will be discussed in more detail after the results of Experiment 4 have been presented.

*Rats placed directly on test after weaning.

Table 1.

Growth response of cows fed the usual dairy

No. of cows in group		34	3	2
No. days in period		49	14	14
Av. initial wt. (grams)		96.7	95.4	77.5
Av. final wt.	"	92.4	79.2	62.5
Total gain	"	4.3	16.2	15.0
Av. daily gain	"	.09	1.16	1.07
Av. daily feed	"	1.57	2.32	1.80

EXPERIMENT 4

Experiments involving the use of pure amino acids lend themselves very favorably to protein quality studies, but they are very expensive. Funds were not available to extend the investigation and since it was considered that the reliability of the results obtained in Experiments 2 and 3 might be questionable in view of the fact that only a small number of test animals was used, the primary purpose of the final trial was to attempt to obtain data which would either substantiate or discredit the results obtained in the preceding trials.

Experimental Material

The grain used, the source, allottment, housing, feeding, weighing of rats and the preparation of rations were the same as in Experiments 2 and 3.

Test Groups

Group No.	Ration
1.	Low protein barley.
2.	Low protein barley + 8% casein.
3.	Low protein barley + lysine in an amount equivalent to that supplied by 8% casein.
4.	Low protein barley + lysine, tryptophane, valine, methionine and threonine in an amount equivalent to that supplied by 8% casein.

5. Low protein barley + 10 essential amino acids in an amount equivalent to that supplied by 8% casein.

Results and Discussion

The average growth response of the rats in the different groups during the 22-day period of the trial is shown in Figure 5 and Table X.

Table X

Growth Response of Rats Fed the Basal Ration Supplemented with Amino Acids

	Group Number				
	1.	2.	3.	4.	5.
	Basal	8% casein	Lysine	5 A. Acids	10 E.A.A.
No. of rats in group	3	3	2*	3	3
No. days in period	22	22	22	22	22
Av. initial wt.**(grams)	40.3	41.3	41.5	40.3	41.3
Av. final wt. "	74.3	129.3	97.5	94.3	121.0
Total gain "	34.0	88.0	56.0	54.0	79.7
Av. daily gain "	1.55	4.00	2.55	2.45	3.62
Av. daily feed "	10.3	12.6	12.5	9.4	10.4
Feed consumed per gram gain (grams)	6.6	3.1	4.9	3.9	2.9

* Three rats were originally included in the group but one became ill on the 6th day of the experiment and was discarded.

** All rats kept on basal ration for two days prior to experiment.

EXPERIMENT 4

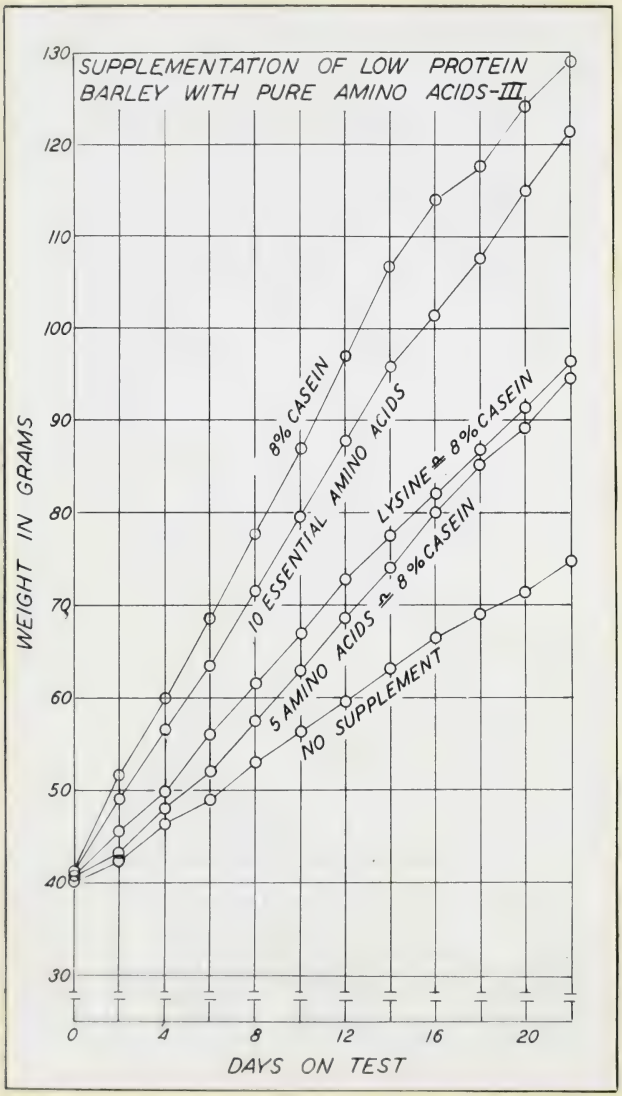

Fig. 5. Growth curves of weanling rats.

If the ration of low protein barley plus 8% casein is assigned a nutritional value of 100, the remaining rations, based on weight attained at the end of experiment, could be ranked as shown below:

Low protein barley + 8% casein	100
Low protein barley + 10 E.A.A.	91
Low protein barley + lysine	64
Low protein barley + 5 A. Acids	61
Low protein barley	39

As in previous experiments, supplemental lysine gave a good response in growth while the addition of ten essential amino acids resulted in almost as rapid growth as was obtained with a supplement of 8% casein.

The inhibitory action shown by a combination of valine, methionine, threonine, tryptophane and lysine was again demonstrated. As has already been indicated, it is difficult to explain why a supplement of lysine alone should be better than a supplement of lysine and tryptophane or of the five amino acids mentioned above. Growth inhibiting effects in rat feeding experiments where pure amino acids have been used as supplements have been reported by some workers and several possible explanations have been offered.

One explanation suggested is the toxic effect of the unnatural forms of amino acids. Albanese and Irby (30) from results obtained in feeding a mixture of ten essential amino acids to rats suggest that growth inhibition on such a diet may be due in part to toxic effects of the unnatural forms of

valine and threonine which cannot be utilized by the rat. Martin (31), on the other hand, reports no evidence of toxicity or nutritive failure in rats fed rations similar to those employed by Albanese and Irby, but containing approximately 10% less dl-valine. The growth inhibiting action of dl-norleucine demonstrated by Womack and Rose (32) did not appear to be due to the unnatural form of norleucine nor to a toxic contaminant in the norleucine molecule since in subsequent experiments (25) the resolved d- and l- forms manifested the same growth inhibitory action as the original dl-norleucine. In recent experiments Frazier and his associates (33) observed no apparent toxic effect when a mixture containing unnatural forms of isoleucine, methionine, phenylalanine, threonine, tryptophane, alanine and aspartic acid was fed to rats.

In view of these findings and the fact that in our experiments the racemic forms of valine, threonine and methionine did not show any inhibitory growth action when included in a supplement of ten essential amino acids, it appears that the deleterious effects shown with a supplement of five amino acids (including the racemic valine, threonine and methionine) are not likely to have been due to the toxic effects of unnatural isomers fed in our ration. Furthermore, the slight inhibitory effect which was observed on the addition of tryptophane to lysine cannot be attributed to unnatural isomers since the natural forms of both these amino acids were used.

Another possible explanation for poorer growth responses when certain amino acids are added to a basal diet may be in the creation of an amino acid imbalance which interferes with growth. Sullivan, Hess and Sebrell (34) obtained excellent growth responses by adding 0.5% cystine to a low protein diet, but a significant retardation in growth when it was added at a 5% level. Hier, Graham and Klein (35) have shown that 50% casein in the diet for the rat has no deleterious effect. They indicate, however, that growth inhibition results from the addition of 6% glycine, or 5% l-proline, or 5% dl-phenylalanine singly to a ration containing 20% casein. These workers also showed that rats fed a mixture of ten essential amino acids in amounts equivalent to Rose's requirements (24) grew better than those on a ration containing 20% gelatin plus the same amino acids, despite the fact that gelatin supplied non-essential amino acids. They suggest that the growth inhibitory effects are due to marked amino acid imbalances and warn of the danger of imbalancing amino acid proportions by introducing high levels of some amino acids even though they may be essential ones.

Recently Groschke and Briggs (36), working with chicks, have observed a marked growth inhibitory effect when arginine, glycine, alanine and gelatin were added in combination to a synthetic ration. The addition of 5 mg. % of nicotinic acid overcame the ill effects of this amino acid mixture and Groschke and Briggs conclude that nicotinic acid is concerned, in some

manner with the metabolism of amino acids. Even if it is agreed that an amino acid imbalance may increase the nicotinic acid requirement of the rat, it does not seem probable that a nicotinic acid deficiency existed in any of the rations used in the present studies, since the calculated nicotinic acid content of these rations is over 7 mg. per cent, which, so far as is known, is well above the dietary requirement of the rat.

From the above discussion it is apparent that growth inhibition associated with the feeding of amino acids is not uncommon and that complete understanding of this phenomenon must await further experimentation. Such experimentation may be expected to add materially to the understanding of protein metabolism and to provide valuable practical information with respect to the dietary use of amino acids.

SUMMARY

1. Grains grown in different areas were shown to vary markedly in nutritional adequacy with respect to protein.

2. As measured by rat growth during an eight week test, barley and oat proteins were found to be superior to wheat proteins.

3. In two experiments rats were fed a basal ration of low protein barley supplemented with the ten amino acids known to be essential for the rat and in both experiments these rats

made almost as rapid gains and economical use of their food as did control rats fed the same basal ration supplemented with 8% casein.

4. In three out of three experiments rats fed a basal ration of low protein barley supplemented with the amino acid lysine made more rapid gains and required less food per gram gain than did control rats fed the unsupplemented basal ration.

5. A supplement of lysine plus tryptophane was used in one trial. This supplement was not as effective as a supplement of lysine alone.

6. A supplement made up of lysine, tryptophane, methionine, threonine and valine was tested in two trials. Rats fed this supplement made slightly less rapid growth than did rats supplemented with lysine alone.

7. The phenomenon of growth inhibition associated with the feeding of certain combinations of amino acids is discussed.

PART II - AMINO ACID ASSAYS ON GRAIN

Evidence of variations in the nutritional value of grains grown under different climatic and soil conditions together with the results of preliminary rat feeding experiments involving the supplementation of a barley ration with amino acids has been presented in Part I. Since grains provide the major portion of the protein ingested by farm animals and poultry, the agricultural importance of data on the quality of grain proteins is evident. However feeding trials designed to study protein quality are very expensive even when small experimental animals are used.

The recent development of microbiological methods of assay provides a new and relatively inexpensive means of obtaining data on the amino acid composition of grains. Since amino acid composition determines protein quality it therefore seemed logical to discontinue the animal feeding trials in favor of initiating amino acid assay studies which offered promise of yielding data on the amino acid pattern of grains which would be fundamental to future animal experimentation.

Microbiological assays done to determine the valine, leucine, isoleucine and phenylalanine content of grains are described in Part II of this thesis. A brief review of literature precedes the presentation of the experimental data.

REVIEW OF LITERATURE

Methods for the determination of amino acids in proteins and natural foodstuffs have been discussed in some detail by Block and Bolling (17), Block and Mitchell (37), Snell (38) and briefly by the author in another paper (39). Four general methods have been used, namely, chemical, physico-chemical, biological and microbiological. Since the work reported in this section deals with microbiological assays, the literature relative to this method of assay, especially as applied to the amino acids valine, leucine, isoleucine and phenylalanine will be briefly reviewed.

Assay Methods

Since 1943 there have been extensive developments in the field of microbiological methods of assay for amino acids. The first published report was that of Kuiken and associates (40) who reported assays on proteins and foodstuffs for leucine, isoleucine and valine using Lactobacillus arabinosus 17-5 as the assay agent. Later McMahan and Snell (41) determined the valine content of proteins and foodstuffs using Lactobacillus arabinosus 17-5 and Lactobacillus casei. Good results were obtained with both organisms.

In 1945, Stokes *et al.* (16) described a method for the assay of the ten essential amino acids applicable to foodstuffs, natural products, purified proteins and synthetic amino acid mixtures. Nine of the amino acids, including valine, leucine

and isoleucine were determined with Streptococcus faecalis, while the tenth, phenylalanine, was assayed with Lactobacillus delbrückii LD5. The latter organism was used for phenylalanine since these workers found that Streptococcus faecalis is able to synthesize this amino acid at a slow rate. Hegsted (43) and Riesen and associates (44) have successfully employed Lactobacillus arabinosus 17-5 as an assay organism for phenylalanine.

Baumgarten, Stone and Boruff (45) determined essential and non essential amino acids in grain alcohol fermentation by-products using Lactobacillus arabinosus 17-5 and Lactobacillus casei. By a modification of the medium, Baumgarten, Mather and Stone (18) developed a technique whereby Streptococcus faecalis R could be used successfully. Lactobacillus casei was used by these workers to assay for phenylalanine. The response of Streptococcus faecalis R, as well as that of Lactobacillus arabinosus 17-5 and Lactobacillus casei to different optical forms of leucine, valine and other amino acids was studied. Assay values for a large number of foodstuffs, including cereal grains, are given.

Hier et al. (46) determined valine, leucine, isoleucine and phenylalanine in animal proteins by the usual microbiological technique of adding 5 cc. of basal medium to the test tube and making the final volume with distilled water to 10 cc. after the addition of the hydrolysate (40, 47). Recently, Hier and Bergeim (48) determined leucine, isoleucine and valine in dog plasma by modifying this earlier technique to permit the use of

1 cc. of the basal medium and the adjustment to a final volume of 2 cc. All other details were carried out as in the macro method except that the tubes were titrated with 0.02 N NaOH. Recent reports of Hier, Cornbleet and Bergeim (49) and Riesen and associates (44) show that the semi-micro technique is accurate and advantageous as compared to the macro procedure.

Regnery (50) has observed that unhydrolyzed casein may be assayed for leucine by means of a leucineless mutant of Neurospora. This line of study has apparently not been extended. Successful development of such a method of assay would solve the problem of amino acid losses in humin during the process of hydrolysis.

Hydrolysis Methods

The methods for protein hydrolysis are discussed in detail by Block and Bolling (17) and are mentioned only briefly here. Enzymes, alkalies or acids are used as hydrolysis agents.

Proteolytic enzymes are used to avoid destruction and racemization of the amino acids, but such hydrolysis seldom goes to completion. In addition the long incubation time required and the possibility that the enzyme may furnish some of the amino acid under assay are disadvantages. Hydrolysis with strong alkalies results in racemization of amino acids and in destruction of some; e.g. cystine, serine and threonine (17).

Acid has been found to be the most satisfactory hydrolysis agent except when tryptophane and tyrosine are under assay. Various acids and methods have been used in hydrolysis of plant

proteins and grains. Kuiken et al. (40) hydrolyzed wheat samples by refluxing with 5 N H_2SO_4 for 24 hours. Schweigert and associates (47) report that autoclaving for 5 hours with 2 N HCl or refluxing for 24 hours with 2 to 4 N HCl or 5 N H_2SO_4 gives cereal and legume hydrolysates satisfactory for microbiological assay. Stokes et al. (16) used 10% HCl autoclaving for 10 hours in sealed ampuls, while Baumgarten, Mather and Stone (18) found that refluxing cereal samples for 24 hours with constant boiling HCl or for 20 hours with 20% HCl proved satisfactory.

EXPERIMENTAL

Assay Materials

Grain samples collected for a research project in progress at the University of Alberta under the title, "Soil and Plant Relations Affecting the Nutritional Value of Foods and Feeds," constituted the assay material. These samples were chosen to represent a wide range in protein content as well as different soil zones.

Organism and Inoculum

Lactobacillus arabinosus 17-5, obtained from the American Type Culture Collection, Georgetown University School of Medicine, Washington, D.C,, was used in the determinations of valine, leucine, isoleucine and phenylalanine described in this paper. The organism was maintained by weekly transfers as stabs in a solid medium containing the following ingredients:

Salts B*	0.5 cc.
K_2HPO_4**	0.5 cc.
Vitamin solution*	2.0 cc.
Glucose	2.0 g.
Tryptone	1.0 g.
Yeast extract	0.5 g.
Sodium acetate	1.0 g.
Agar	2.0 g.

Distilled water to 100 cc. final volume.

To prepare the inoculum, cells from the agar stabs were transferred to a liquid medium of the same composition except for agar and cultured for 24 hours at 37°C. The liquid cultures were centrifuged and the medium was decanted thoroughly. As a final step the cells were resuspended in approximately 30 cc. of sterile physiological saline. Aseptic technique was practised throughout.

Basal Medium

The composition of stock solutions for the basal medium used for the determination of valine, leucine, isoleucine and phenylalanine employing Lactobacillus arabinosus 17-5 as the assay agent is presented in Table XI. This synthetic medium is similar to that employed by Riesen, Schweigert and Elvehjem (51) for the same organism except for the following changes:

*Stock solutions, see "Basal Medium".
**Stock solution of same concentration as salts A, see "Basal Medium".

(a) l(-) Cystine, l(+) arginine monohydrochloride, l(+) histidine hydrochloride, l(-) tyrosine and dl-tryptophane were doubled in quantity (44) in order to standardize the medium for the three organisms, Lactobacillus arabinosus 17-5, Streptococcus faecalis R and Leuconostoc mesenteroides PD-60. The latter two organisms were used by other workers in the laboratory.

(b) Xanthine was included in the medium (44).

(c) Pyridoxamine was used in place of pyridoxine (52).

(d) Choline and inositol were omitted (44).

(e) Pyridoxamine dihydrochloride and p-aminobenzoic acid were added in somewhat smaller quantities (58).

Table XI

Composition of Stock Solutions for Basal Medium for Valine, Leucine, Isoleucine and Phenylalanine Assay.

Solution 1

(Amino Acids)

l(-) Leucine*	200 mg.	Dissolve in about 10 cc. hot 2 N HCl, and dilute to 100 cc. with distilled water.
dl-Isoleucine*	400 "	
dl-Valine*	400 "	
l(-) Cystine	400 "	
dl-Methionine	400 "	
l(-) Tryptophane	100 "	
l(-) Tyrosine	200 "	
dl-Phenylalanine*	200 "	
l(+) Glutamic acid	800 "	
dl-Threonine	400 "	
dl-α-Alanine	400 "	
dl-Aspartic acid**	800 "	
l(+) Lysine monohydrochloride	400 "	
l(+) Arginine monohydrochloride	200 "	
l(+) Histidine monohydrochloride	200 "	

* Appropriate amino acids omitted from the basal medium.

** Aspartic acid purchased from Merck and Co. Inc., Rahway, N. J. All other amino acids purchased from General Biochemicals, Chagrin Falls, Ohio.

Solution 2

(Purines and Pyrimidines)

Adenine sulfate + $2H_2O$	200 mg.	Solution effected by prolonged heating in a few drops of HCl plus a small amount of water. Dilute with distilled water to 200 cc. final volume.
Guanine hydrochloride + $2H_2O$	200 "	
Uracil	200 "	

Solution 3

(Xanthine)	100 mg.	Dissolve in small quantity of concentrated NH_4OH and make up to 100 cc. with distilled water.

Solution 4

(Vitamin solution)

Thiamine hydrochloride	12.5 mg.	Dissolve by heating in 250 cc. distilled water.
Riboflavin	12.5 "	
Niacin	25.0 "	
dl-Ca pantothenate	12.5 "	
Pyridoxamine dihydrochloride	5.0 "	
p-Aminobenzoic acid	2.5 "	
Biotin	25 micrograms	
Folic acid	250 "	

Solution 5

(Salts A)

KH_2PO_4	25 g.	Dissolve by warming in 250 cc. distilled water.
K_2HPO_4	25 g.	

Solution 6

(Salts B)

$MgSO_4 \cdot 7H_2O$	5 g.	Dissolve by warming in 125 cc. distilled water.
NaCl	0.25 g.	
$FeSO_4 \cdot 7H_2O$	0.25 g.	
$MnSO_4 \cdot 4H_2O$	0.25 g.	

Ingredient 7

(Glucose)

Ingredient 8

(Sodium acetate)

See Table XII.

The basal medium was prepared from stock solutions which were stored in the refrigerator. The amounts of each of the constituents used to make up sufficient basal medium for 100 assay tubes are shown in Table XII.

Table XII

The Quantity of Stock Solutions used to prepare Basal Medium Sufficient for 100 test tube Assay

(Semi-Micro Scale)

Constituent	Amount Used
Glucose	4 grams
Sodium Acetate	4 grams
Amino Acids	10 cc.
Purines and Pyrimidines	2 cc.
Xanthine	2 cc.
Vitamin solution	2 cc.
Salts A	1 cc.
Salts B	1 cc.

About 50 cc. of distilled water were added to this mixture to dissolve the glucose and sodium acetate. The pH of this solution was then adjusted to 6.8 using a spot plate and brom-thymol blue indicator. The final volume was made to 100 cc.

Preparation of Samples for Assay

One gram samples of grain which had been ground to pass through the 1 mm. screen of a Wiley Mill were autoclaved in 250 cc. Erlenmeyer flasks with 25 cc. of 2 N HCl for 10 hours at 15 lb. pressure. Erlenmeyers were covered with 50 cc. beakers. The samples were cooled to room temperature and made up to 100 cc. in volumetric flasks. They were then

Table III

Constituent	Amount used
Glucose	[illegible] grams
Sodium Acetate	[illegible] grams
Agino acids	10 cc.
Purines and Pyrimidines	2 cc.
Xanthine	2 cc.
[illegible]	[illegible] cc.
Salts A	1 cc.
Salts B	1 cc.

About 70 cc. of distilled water were added to this mixture to dissolve the glucose and amino acids. The pH of this solution was then adjusted to 6.4 using a dilute sulphuric-butyral blue indicator. The final volume was made to 100 cc.

Preparation of standing Cell Layer

[illegible] cc. aliquots of [illegible] were pipetted into [illegible] cc. Erlenmeyer flasks with 25 cc. of 5 % NaCl and centrifuged at 15 lb. pressure. Supernatant was removed with [illegible] cc. pipettes. The samples were cooled to room temperature and made up to 100 cc. in volumetric flasks. They were then

filtered through coarse filter paper and the humin was discarded. A 20 cc. aliquot of the filtrate was brought to a pH of 6.8 with 14 N NaOH using a Beckman pH meter. The neutralized filtrate was then diluted to 100 cc. in a volumetric flask. Fifty cc. of this 1:500 dilution were retained for valine, leucine and isoleucine assay. A 10 cc. aliquot of the remainder was diluted to 20 cc. to make a 1:1000 dilution for phenylalanine assay.

Assay Procedure

Assays were conducted on a semi-micro scale in which 1 cc. of basal medium was used and the final volume was adjusted to 2 cc. with water (44, 48, 49). The basal medium and water in appropriate amounts were added with a Cannon automatic dispensor (42) to 20 x 150 mm. bacteriological test tubes. A 1 cc. pipette graduated in tenths was used to add hydrolysates which had been prepared and diluted as previously described. After considerable experience 3 levels of the hydrolysate in duplicate were used; 0.2, 0.4 and 0.6 cc. for leucine, and 0.4, 0.6 and 0.8 cc. for valine, isoleucine and phenylalanine.

The tubes, in racks provided with metal covers, were autoclaved for 15 minutes at 15 lb. pressure. After cooling, each tube was inoculated with 1 drop of the cell suspension previously described. After incubation at 37°C. for 72 hours, the lactic acid produced was titrated directly in the tubes by means of a suitable burette equipped with a titrating

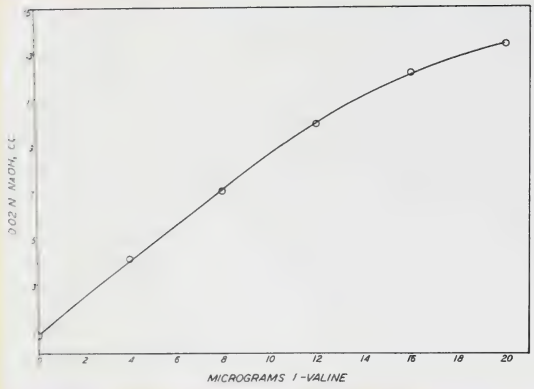

Fig. 6. Standard curve for l-valine.

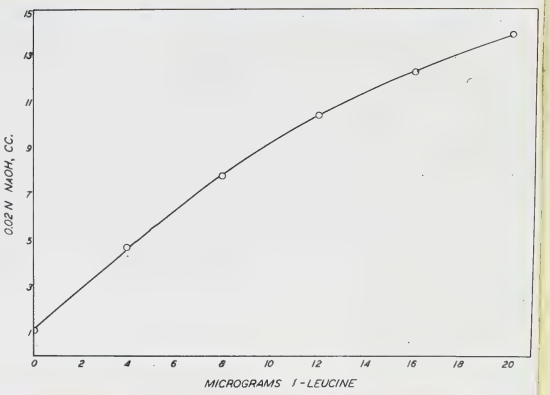

Fig. 7. Standard curve for l-leucine.

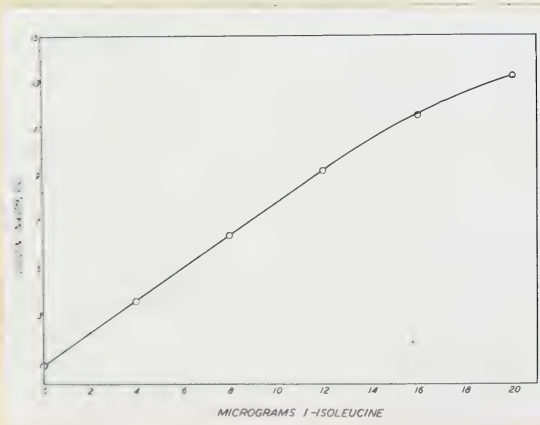

Fig. 8. Standard curve for l-isoleucine.

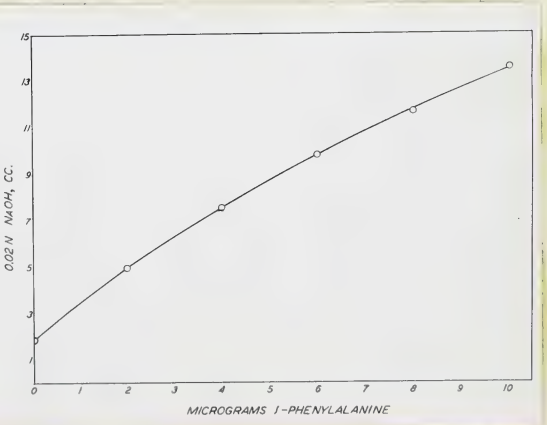

Fig. 9. Standard curve for l-phenylalanine.

device designed by Lunardelli et al.(53). Brom-thymol blue was used as the indicator, and 0.02 N NaOH was used as the base. From the volume of the base used to neutralize the acid produced in the test tubes containing the unknown hydrolysate, the amino acid content was readily calculated from the standard curve.

Preparation of Standards

Separate stock solutions of the natural forms of valine, leucine, isoleucine and phenylalanine were prepared at an exact concentration of 0.5 mg. per cc. and were stored in the refrigerator. A suitable aliquot of the stock solution of phenylalanine was taken, adjusted to pH 6.8 and diluted to a concentration of 10 micrograms per cc. with distilled water. Valine, leucine and isoleucine were diluted in a similar way to a concentration of 20 micrograms per cc. Titration results obtained from a series of tubes treated in the same manner as those containing the test hydrolysates, but made up in triplicate with 0.2, 0.4, 0.6, 0.8 and 1.0 cc. of the above amino acid solutions, were used to prepare standard curves from which the amino acid equivalents of the test hydrolysates could be read.

Typical standard curves for the four amino acids are shown in Figures 6, 7, 8 and 9.

Validity of Procedure

Hydrolysis Agents

The use of acid as a hydrolysis reagent for the liberation of valine, leucine, isoleucine and phenylalanine for microbiological assay has been reported by a number of workers (17, 40, 44, 47). The possibility of using enzymatic hydrolysates in amino acid assay studies has also been suggested (17, 44). In the present study only a limited amount of work was done to compare different hydrolytic agents. The agents tested and the results obtained are shown in Tables XIII and XIV.

Table XIII

Agents and Methods of Hydrolysis

	Wt. of Sample	Agent	Quantity	Method
(1)	1 g.	2 N HCl	25 cc.	Autoclave 10 hr. 15 lb. pressure
(2)	1 g.	6 N HCl	25 cc.	Reflux, 24 hr.
(3)	1 g.	Pancreatin*	75 mg.	Shaken for 80 hr. at 37°C.
		+ Intestinal Mucosa	25 mg.	
(4)	1 g.	Ficin*	100 mg.	Shaken for 80 hr. at 37°C.

*1 gram grain samples were placed in 100 cc. volumetric flasks and the specified amounts of proteolytic enzymes, together with 50 cc. of 0.2 M buffer ($Na_2HPO_4 \cdot 12\ H_2O$) were added to the flasks. One cc. of toluene was also added to the flasks to prevent bacterial growth during digestion.

Table XIV

Assay Results Obtained on Acid and Enzyme Hydrolysates of Wheat (#5-W-1)

Hydrolysis Agent	Assay values in mg/gram wheat. Valine	Leucine	Isoleucine	Phenylalanine
2 N HCl	9.75	15.00	10.65	11.83
6 N HCl	9.80	15.00	10.55	11.75
Pancreatin + Intestinal Mucosa	8.05	12.93	8.24	8.64
Ficin	4.70	8.10	5.05	5.35

Ficin, a very active proteolytic enzyme of plant origin, released the smallest amount of the four amino acids. The combination of pancreatin and intestinal mucosa, although more efficient than ficin was not as effective as acid hydrolysis. Greenhut, Schweigert and Elvehjem (54) have recently found that a mixture of pancreatin and intestinal mucosa was effective in releasing tryptophane from a number of proteins and suggest that particle size may be a very important factor in determining the rate and completeness of hydrolysis. The results shown in Table XIV are for a sample of grain ground to pass through a 1 mm. screen and it may be that finer grinding is necessary in order to obtain complete hydrolysis by enzymatic methods.

There was no appreciable difference in the yield of amino acids obtained by the two methods of acid hydrolysis tested. The method of autoclaving in 2 N HCl is more rapid

than refluxing in 6 N HCl and offers an additional advantage in that the neutralized hydrolysate used for assay contains less salt which might inhibit bacterial growth. In view of these advantages, autoclaving in 2 N HCl was adopted as the hydrolysis procedure in the present study.

Duration of Autoclaving in 2 N HCl

The relation between duration of autoclaving and the degree of hydrolysis of cereal grain proteins was studied. The liberation of α-amino nitrogen as determined by the micro-nitrous acid method of Van Slyke (55) was used as the criterion of the completeness of hydrolysis. The percent of α-amino nitrogen liberated from wheat, oats and barley at intervals between one and ten hours is shown in Figure 10.

From the curves shown in Fig. 10 it is apparent that maximum hydrolysis of all three grains was obtained after approximately seven hours. Since the level of α-amino acid nitrogen remained constant at intervals between seven and twenty hours, autoclaving for ten hours was adopted as a routine procedure which would assure maximum hydrolysis.

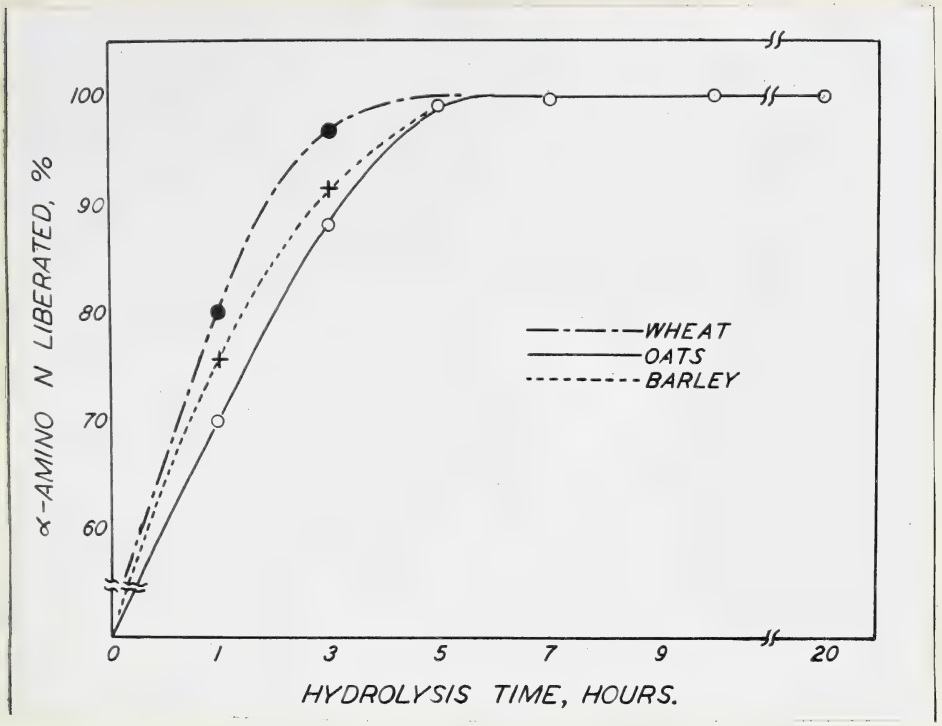

Figure 10. Effect of duration of autoclaving on the α-amino nitrogen content of hydrolysates of wheat, oats and barley.

Losses of Amino Acids in Humin

In the acid hydrolysis of proteins a dark brown or black precipitate of humin is formed. There is evidence to show that tryptophane and tyrosine, in the presence of carbohydrates are concerned in humin formation (19, 56). Kuiken et al. (40) have shown that some losses of leucine, isoleucine and valine do occur as a result of humin formation but they are not great enough to seriously effect the usefulness of determinations of these amino acids in the proteins of natural foodstuffs. Roxas (57) has shown that leucine and phenylalanine

are not factors in humin formation.

Two experiments were done to determine the amount of nitrogen lost in humin during hydrolysis of grains by autoclaving in 2 N hydrochloric acid. As shown in Table XV losses of 5.9 to 8.5 per cent were detected.

Table XV

Losses of Nitrogen in Humin when Grains were Autoclaved in 2 N HCl for 10 Hours.

Sample No.	Wt. Sample	Grain N in mg.	Humin N in mg.	% N lost
5-B-3	1 gram	17.4	1.34	7.7
5-B-1	"	31.6	2.68	8.5
5-O-3	"	17.7	1.41	8.0
5-O-1	"	30.1	2.26	7.5
5-W-3	"	18.7	1.11	5.9
5-W-1	"	40.0	2.80	7.0

Salt Inhibitory Effects

Riesen, Schweigert and Elvehjem (51) in agreement with Dunn et al. (6) detected no inhibitory effects with Leuconostoc mesenteroides PD-60 at salt concentrations up to 40 mg. per tube. However, at a concentration of 15 mg. NaCl per tube decreased acid production with Lactobacillus arabinosus 17-5 and Streptococcus faecalis was observed (51). The maximum amount of NaCl that was present in the grain hydrolysates used

are not [illegible] in [illegible] conversion.

[illegible] were done by [illegible] nitrogen [illegible] in [illegible] [illegible] clearing in 2 % hydrochloric acid. [illegible] [illegible] of 5.9 to 6.5 per cent were determined.

Table 17

Losses of nitrogen in sugary sugar [illegible] were [illegible]

Sample no.	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

[illegible] [illegible] [illegible] [

in this study was approximately 2 mg. per tube, which is well below the inhibitory level.

Reproducibility.

Table XVI shows typical results obtained in tests on wheat and barley when three separate assays were done for the four amino acids. Assays 1 and 2 were done on the same hydrolysate on different days, while assay 3 was carried out on a new hydrolysate of the same samples also on a different day.

Table XVI

Comparison of Results of Three Separate Assays on Wheat and Barley

Amino acid	Assay 1	Assay 2	Assay 3	Mean and max. dev. from the mean	Max. % dev.
			Wheat (5-W-9)		
Valine	7.5	7.6	7.8	7.6 ± 0.2	2.6
Leucine	11.7	11.9	11.5	11.7 ± 0.2	1.7
Isoleucine	7.9	8.0	8.5	8.1 ± 0.4	4.9
Phenylalanine	9.6	9.9	10.5	10.0 ± 0.5	5.0
			Barley (5-B-1)		
Valine	8.6	8.4	9.1	8.7 ± 0.4	4.6
Leucine	11.3	11.8	10.8	11.3 ± 0.5	4.4
Isoleucine	7.8	7.0	7.1	7.3 ± 0.5	6.8
Phenylalanine	8.6	8.6	8.8	8.7 ± 0.1	1.1

below the Inhibitory Level.

Table XVI shows typical results obtained in tests on wheat and maize, when three separate samples were used for the four media. Samples I and 2 were from one consignment on a new consignment of the same samples also on a different day.

Table XVI

Comparison of Results on Three Separate Samples on

The maximum percentage deviation from the mean shown in Table XVI is 6.8. In routine determinations assays were repeated three times and results which deviated from the mean by more than 10% were discarded.

Recovery Experiments.

Stability of the individual amino acids under the conditions of hydrolysis was investigated by adding known quantities of amino acids to grain samples prior to hydrolysis and determining the percentage recovery of each. Recoveries ranged from a low of 88% to a high of 114% while the mean was 97% for valine, 101% for leucine, 99% for isoleucine and 95% for phenylalanine. Typical results are shown in Table XVII.

Table XVII

Recovery of Amino Acids Added to

Grain Samples prior to Hydrolysis.

Grain	Amino Acid	Amino Acid in grain mg/g.	Amino Acid added mg/g.	Amino Acid Recovered mg/g.	Recovery %
Wheat 5-W-1	Valine	9.8	10.0	18.8	90
	Leucine	12.8	10.0	24.0	112
	Isoleucine	9.5	10.0	19.9	104
	Phenylalanine	11.8	15.0	26.6	99
Wheat 5-W-2	Valine	6.8	10.0	16.3	95
	Isoleucine	7.2	10.0	17.2	100
	Phenylalanine	8.3	15.0	22.2	93
Barley 5-B-9	Valine	7.4	10.0	18.0	106
	Leucine	10.7	10.0	20.8	101
	Isoleucine	6.1	10.0	16.0	99

RESULTS AND DISCUSSION

The assay values obtained in the first series of determinations done on high, medium and low protein wheat, barley and oats are compiled in Table XVIII. The data indicate that the proportions of the four amino acids in the grains assayed were approximately the same regardless of the protein content of the samples. For example, a 19.7% protein wheat contained roughly twice as much of each of the four amino acids as did a wheat containing 9.2% protein. Further, the type of soil on which grains were grown did not appear to affect the relationship between the level of any of the four amino acids investigated and the protein content of the samples.

Two varieties of barley were assayed and the results shown in Table XVIII do not suggest any marked varietal differences in amino acid pattern, although it may be noted that in terms of percent amino acid nitrogen of total nitrogen both samples of Olli barley were richer in isoleucine content than was the sample of Newal. Further, the corresponding figures for isoleucine in the nine samples of Newal barley listed in Table XX are all somewhat lower than those shown for Olli in Table XVIII. The question of the possibility of differences between varieties in amino acid composition requires study.

Table XVIII

Valine, Leucine, Isoleucine and Phenylalanine Content Of

High, Medium and Low Protein Wheat, Barley and Oats, Grown in Alberta.

Sample No.[1]	Variety	Soil Zone[2]	Station	Nitrogen %[3]	Protein %[4]	(1) % amino acid in whole grain.[5] (2) % amino acid N of total N. Valine (1)	Valine (2)	Leucine (1)	Leucine (2)	Isoleucine (1)	Isoleucine (2)	Phenylalanine (1)	Phenylalanine (2)
5-W-1	Marquis	L.B.	Conrad	4.00	19.7	0.95	3.15	1.41	4.18	1.01	3.00	1.15	2.73
5-W-2	"	B.	Bremner	2.78	13.7	0.71	3.45	0.96	4.21	0.73	3.17	0.79	2.77
5-W-3	"	G.	Fallis	1.87	9.2	0.50	3.48	0.66	4.17	0.52	3.26	0.55	2.73
5-B-1	Newal	L.B.	Legend	3.16	17.1	0.87	3.58	1.12	4.15	0.72	2.63	0.86	2.50
5-B-2	Olli	B.	Mundare	2.40	13.0	0.71	3.92	0.97	4.79	0.61	2.96	0.72	2.79
5-B-3	"	B.	Stony Plain	1.74	9.4	0.53	3.91	0.61	4.02	0.56	3.68	0.48	2.53
5-O-1	Banner	L.B.	Taber	3.01	16.3	0.80	3.42	1.03	3.95	0.71	2.72	0.73	2.22
5-O-2	"	B.	Clover Bar	2.27	12.3	0.59	3.39	0.72	3.70	0.53	2.69	0.55	2.25
5-O-3	"	G.	Warburg	1.77	9.6	0.52	3.78	0.61	4.01	0.50	2.88	0.45	2.37

1. W = Wheat; B = Barley; O = Oats.
2. L.B. = Light brown; B. = Black; G. = Gray-wooded.
3. Moisture-free basis.
4. Calculated to 13.5% moisture.
5. Air-dry basis.

Carte XVIII

Nombre moyen de jours de gelée blanche sous abri météorologique

(1) Moyenne des années 1931 à 1960 inclus.

In the preceding series of assays only three samples each of wheat, barley and oats were studied. Nine samples each of wheat, barley and oats of one variety were assayed in a second series of determinations. The samples used were grown in a number of different soil zones and represented a wide range of protein content. The origin, together with the nitrogen, protein, valine, leucine, isoleucine and phenylalanine content of these grains is shown in Tables XIX, XX and XXI.

Table XIX

Valine, Leucine, Isoleucine and Phenylalanine Content of Marquis Wheat Grown in Alberta.

Sample No.	Soil[1] Zone	Station	Nitrogen[2] %	Protein %[3] (N x 4.93)	(1) % amino acid in whole grain.[4] (2) % amino acid N of total N. Valine (1)	Valine (2)	Leucine (1)	Leucine (2)	Isoleucine (1)	Isoleucine (2)	Phenylalanine (1)	Phenylalanine (2)
5-W-3	G.	Fallis	1.87	9.2	0.50	3.48	0.66	4.17	0.52	3.26	0.55	2.73
5-W-4	D.B.	Airdrie	2.57	12.7	0.70	3.58	1.01	4.59	0.73	3.31	0.76	2.72
5-W-2	B.	Bremner	2.78	13.7	0.71	3.45	0.96	4.21	0.73	3.17	0.79	2.77
5-W-17	D.B.	Nobleford	2.94	14.5	0.75	3.27	1.11	4.32	0.76	2.96	0.90	2.77
5-W-6	D.B.	Chauvin	3.04	15.0	0.76	3.26	1.15	4.41	0.80	3.09	0.91	2.76
5-W-9	D.B.	Metiskow	3.05	15.0	0.78	3.31	1.16	4.43	0.80	3.05	0.95	2.85
5-W-15	D.B.	Craigmyle	3.15	15.5	0.79	3.24	1.15	4.19	0.80	2.92	0.97	2.79
5-W-14	D.B.	Drumheller	3.32	16.4	0.81	3.22	1.23	4.37	0.85	3.04	1.03	2.92
5-W-1	L.B.	Conrad	4.00	19.7	0.95	3.15	1.41	4.18	1.01	3.00	1.15	2.73

1. G. = Gray-wooded; D.B. = Dark brown; B. = Black; L.B. = Light brown.
2. Moisture-free basis.
3. Calculated to 13.5% moisture.
4. Air-dry basis.

Table XX

Valine, Leucine, Isoleucine and Phenylalanine Content of Newal Barley Grown in Alberta.

Sample No.	Soil[1] Zone	Station	Nitrogen %[2]	Protein %[3] (N x 5.41)	(1) % amino acid in whole grain.[4] (2) % amino acid N of total N.							
					Valine		Leucine		Isoleucine		Phenylalanine	
					(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
5-B-10	G.	Chedderville	1.81	9.8	0.52	3.76	0.67	4.36	0.44	2.82	0.46	2.38
5-B-19	G.	Baldonnel	2.01	10.9	0.58	3.68	0.79	4.48	0.49	2.79	0.56	2.54
5-B-21	G.T.	Debolt	2.09	11.3	0.63	3.88	0.85	4.64	0.51	2.82	0.58	2.54
5-B-25	G.T.	Progress	2.31	12.5	0.68	3.77	0.91	4.55	0.56	2.77	0.67	2.68
5-B-11	B.T.	Dickson	2.48	13.4	0.72	3.75	0.95	4.48	0.59	2.78	0.70	2.62
5-B- 9	D.B.	Metiskow	2.59	14.0	0.73	3.67	1.00	4.44	0.61	2.70	0.73	2.59
5-B-14	D.B.	Pincher Creek	2.77	15.0	0.81	3.68	1.12	4.58	0.66	2.71	0.85	2.80
5-B-20	B.T.	Beaverlodge	2.97	16.1	0.86	3.67	1.20	4.58	0.71	2.73	0.91	2.76
5-B- 1	L.B.	Legend	3.16	17.1	0.87	3.58	1.14	4.18	0.72	2.63	0.86	2.53

1. G. = Gray-wooded; G.T. = Gray transitional; B.T. = Black transitional; D.B. = Dark brown; L.B. = Light brown.
2. Moisture-free basis.
3. Calculated to 13.5% moisture.
4. Air-dry basis.

Table XXI

Valine, Leucine, Isoleucine and Phenylalanine Content of Victory Oats Grown in Alberta.

Sample No.	Soil Zone[1]	Station	Nitrogen %[2]	Protein % (N x 5.41)[3]	(1) % amino acid in whole grain.[4] (2) % amino acid N of total N. Valine (1)	Valine (2)	Leucine (1)	Leucine (2)	Isoleucine (1)	Isoleucine (2)	Phenylalanine (1)	Phenylalanine (2)
5-0-10	G.	Chedderville	1.49	8.1	0.48	4.23	0.56	4.43	0.43	3.36	0.38	2.42
5-0-19	G.	Baldonnel	1.68	9.1	0.55	4.23	0.66	4.52	0.48	3.21	0.44	2.38
5-0-24	G.	Goodfare	2.00	10.8	0.66	4.25	0.81	4.60	0.58	3.30	0.57	2.55
5-0-12	G.	Fallis	2.13	11.5	0.71	4.37	0.87	4.70	0.63	3.43	0.59	2.54
5-0-26	G.T.	Athabasca	2.29	12.4	0.74	4.19	0.90	4.54	0.64	3.28	0.61	2.49
5-0- 7	D.B.	Castor	2.47	13.4	0.81	4.25	1.01	4.81	0.69	3.28	0.69	2.59
5-0- 8	D.B.	Drumheller	2.55	13.8	0.84	4.27	1.08	4.90	0.73	3.33	0.73	2.63
5-0-16	D.B.	Craigmyle	2.75	14.9	0.97	4.55	1.25	5.20	0.81	3.35	0.84	2.76
5-0-13	L.B.	Acadia Valley	3.13	16.9	0.98	3.99	1.26	4.63	0.86	3.13	0.85	2.46

1. G. = Gray-wooded; G.T. = Gray transitional; D.B. = Dark brown; L.B. = Light brown.
2. Moisture-free basis.
3. Calculated to 13.5% moisture.
4. Air-dry basis.

A statistical analysis of the results listed in these three tables shows that a very high correlation exists between the total protein and the valine, leucine, isoleucine and phenylalanine content of the samples. Also, the results of the second series of assays are in agreement with those of the first series indicating that there is no marked difference in the levels of these four amino acids in the protein of grains of one variety grown on different types of soil.

Table XXII

Correlation Coefficients between Protein and Amino Acid Content of the Grains Listed in Tables XIX - XXI.

Grain	No. Samples	Amino Acid	Corr. Coeff.
Marquis Wheat	9	Valine	+ 0.98**
	9	Leucine	+ 0.99**
	9	Isoleucine	+ 0.99**
	9	Phenylalanine	+ 0.99**
Newal Barley	9	Valine	+ 0.99**
	9	Leucine	+ 1.0 **
	9	Isoleucine	+ 1.0 **
	9	Phenylalanine	+ 0.98**
Victory Oats	9	Valine	+ 0.99**
	9	Leucine	+ 0.98**
	9	Isoleucine	+ 0.98**
	9	Phenylalanine	+ 0.96**

** Significant beyond 1% point.

From Tables XIX - XXI the mean values and standard deviations for valine, leucine, isoleucine and phenylalanine expressed as per cent amino acid nitrogen of total nitrogen in each of the three grains are:

	Marquis Wheat	Newal Barley	Victory Oats
valine	3.33 ± 0.14	3.72 ± 0.09	4.26 ± 0.15
leucine	4.32 ± 0.14	4.48 ± 0.14	4.70 ± 0.23
isoleucine	3.09 ± 0.13	2.75 ± 0.02	3.30 ± 0.09
phenylalanine	2.78 ± 0.06	2.60 ± 0.13	2.54 ± 0.11.

From this data it would appear that oat proteins and possibly barley proteins are richer in valine and leucine than is wheat protein.

However, it must be stressed that from Table XVIII, mean values of 3.53, 3.88, 2.76 and 2.28 per cent amino acid nitrogen of total nitrogen may be calculated for valine, leucine, isoleucine and phenylalanine respectively in Banner oats. These values, when compared with those given above for Victory oats, suggest that intervarietal differences may be as great or greater than differences between species. Comparable data for all the essential amino acids in a number of varieties of each of these three grains might provide an explanation of the results of Experiment I, Part I, in which it was found that the weight gains of rats fed wheat were poor in comparison to those of rats fed either oats or barley.

Baumgarten, Mather and Stone (18) have reported amino acid values for single samples of wheat, barley and oats assayed microbiologically. A comparison of their results and ours on samples of approximately the same nitrogen content is given below. The values shown are in terms of per cent amino acid of moisture free grain.

	Alberta			Baumgarten et.al.		
	Marquis Wheat	Newal Barley	Victory Oats	Wheat	Barley	Oats
Nitrogen, %	2.57	2.77	2.55	2.6	2.9	2.7
Valine	0.77	0.86	0.91	0.7	0.7	0.7
Leucine	1.10	1.19	1.17	1.0	1.0	1.1
Isoleucine	0.79	0.70	0.79	0.7	0.7	0.7
Phenylalanine	0.83	0.91	0.79	0.7	0.7	0.8

From the above data it is apparent that in general our results are slightly higher than those obtained by Baumgarten, Mather and Stone on grain of comparable nitrogen content. It may also be noted that the values reported by Baumgarten and his associates for single samples of wheat, oats and barley of unspecified variety do not suggest the possibility of species differences with respect to these four amino acids. It has been mentioned that our results suggest the possibility of both species and varietal differences in the amino acid composition of grains.

SUMMARY

1. Microbiological assays for valine, leucine, isoleucine and phenylalanine in grain hydrolysates have been described.

2. The level of each of these amino acids was found to vary directly with the level of total protein in wheat, barley and oats.

3. Evidence indicating the possibility of species and varietal differences in the amino acid composition of grains has been presented.

4. Although the type of soil on which wheat, barley or oats is grown has an influence on the total protein content of these grains and thus on the absolute levels of valine, leucine, isoleucine and phenylalanine, the data obtained in this study do not indicate that there is any correlation between soil type and protein quality as measured by the proportion of these four amino acids in grains grown in different soil zones.

EXPERIMENT 1

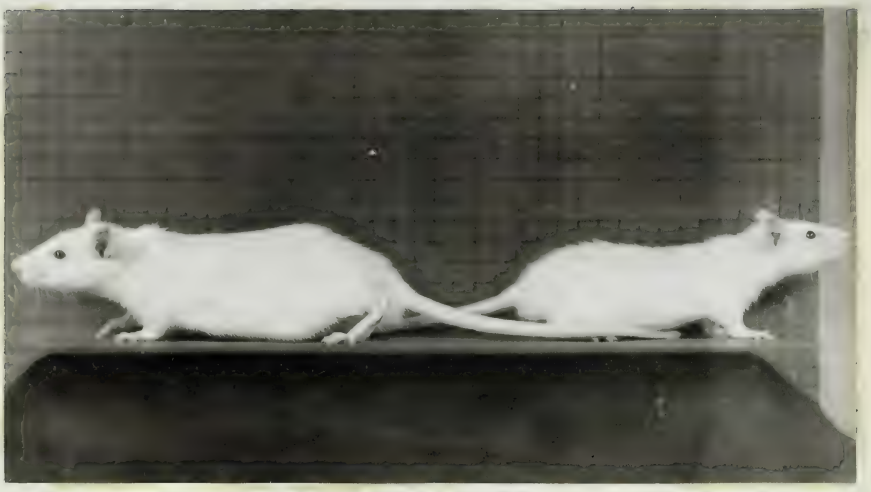

Plate 1. The rat on the left was fed barley containing 16.2% protein, while the one on the right was fed a 9.4% protein barley. Age 59 days.

EXPERIMENT 2

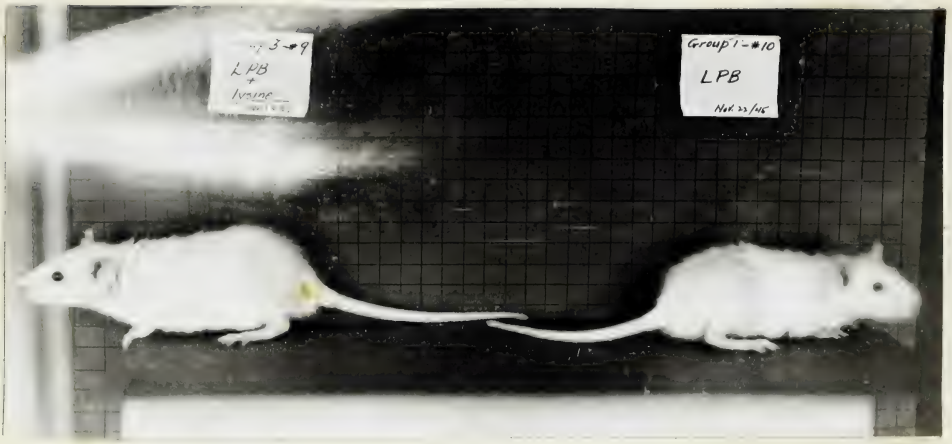

Plate 2. The rat on the left received a supplement of lysine. The rat on the right was fed the basal ration of low protein barley. Age 45 days.

EXPERIMENT 2

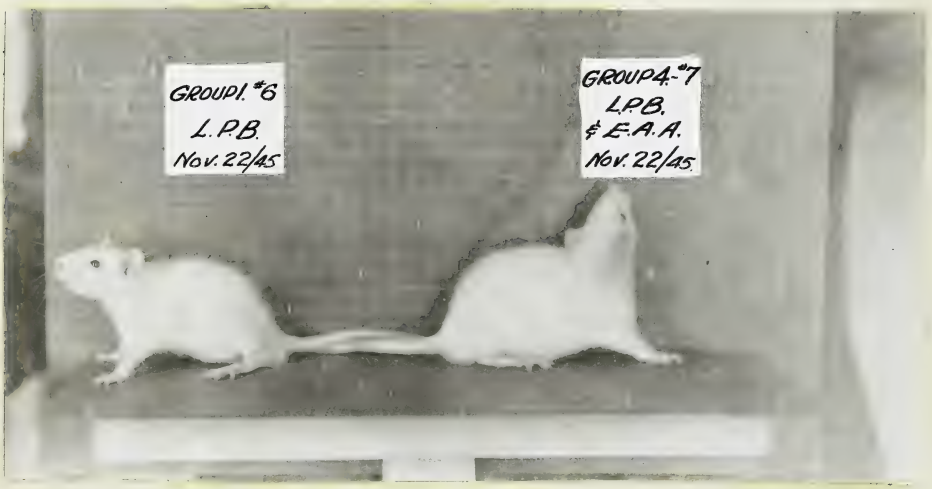

Plate 3. The rat on the left was fed the basal ration of low protein barley. The rat on the right received a supplement made up of the ten amino acids which are known to be essential for rat growth. Age 45 days.

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